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FULL PAPER

Sustainable synthesis of *N*-methylated peptides in a continuous-flow fixed bed reactor

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Abstract

A rapid, simplified and highly efficient continuous-flow solid-phase peptide synthesis technology is reported for the direct synthesis of mono and multiple *N*-methylated cyclic alanine and valine peptides. Through an optimization study, we find that only 1.5 equivalents of the amino acids are sufficient for the couplings to maintain excellent conversions. Importantly, the technology is outstandingly sustainable, since three chemical steps are cancelled from the procedure and low amount of solvent is used, compared to traditional technologies. Furthermore, it is also applicable to the coupling of challenging amino acids, since pentavalines were constructed with high yield. The technology was successfully upscaled and peptide cyclization was carried out too.

Keywords peptides · synthesis · peptidomimetics · continuous-flow · SPPS · *N*-methylation

Introduction

Studying peptides has been the focus of research for many years, because of the large variety of application of these biomolecules in a number of fields, including rational drug development [1–4], nanotechnology [5–8], material science [9–12], etc. However, there are serious limitations in the use of peptides in medicine. Major obstacles are metabolic instability of peptides owing to fast degradation by the proteases and peptidases in the systemic circulation of the human body (short biological half-life) and poor oral bioavailability [13–15]. Another great drawback of peptides is their poor membrane permeability [16]. One of the synthetic modifications to depress enzymatic cleavage and to insure facile absorption into the systemic blood circulation is the methylation of peptide backbone [17–19]. Peptides containing *N*-methyl amino acids appear regularly in nature: in plants, marine sponges and numerous microorganisms, for example Cyclosporine A, with interesting pharmacological profile and therapeutic potential [20, 21]. The importance of *N*-methylated peptides is warranted by further significant biomedical applications too [22, 23].

Multiple *N*-methylated peptides are reluctantly applied [24] possibly due to the cumbersome and often failed couplings of the amino acids onto sterically hindered *N*-methylated sites [25] and incalculable conformational changes [26, 27]. *N*-methylated peptides are mainly synthesized in an indirect way by the on-resin methylation of ordinary α-amino acids. The reason is the relatively high price of Fmoc-protected *N*-methylated amino acids, which excludes their direct utilization thereof. Nonetheless, the in situ methylation requires three additional time-consuming steps, extra reagents and produces excess amount of waste. Moreover, during Fmoc removal epimerization and formation of diketopiperazine [25] can occur. Even more problematic is the spontaneous deprotection of the Fmoc-amino acid. This is caused by the secondary amine functional group of the N-terminus resulting in the additional incorporation of amino acids [28].

Continuous flow (CF) techniques have recently emerged as a productive methodology in modern synthetic chemistry. This is due to the substantial number of advantages against conventional batch procedures, such as faster heat and mass

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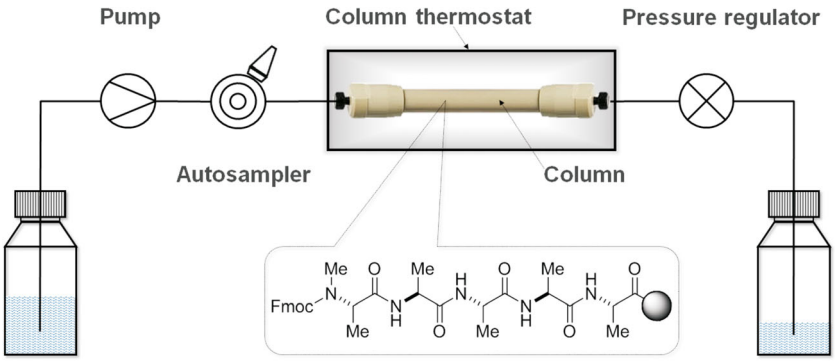
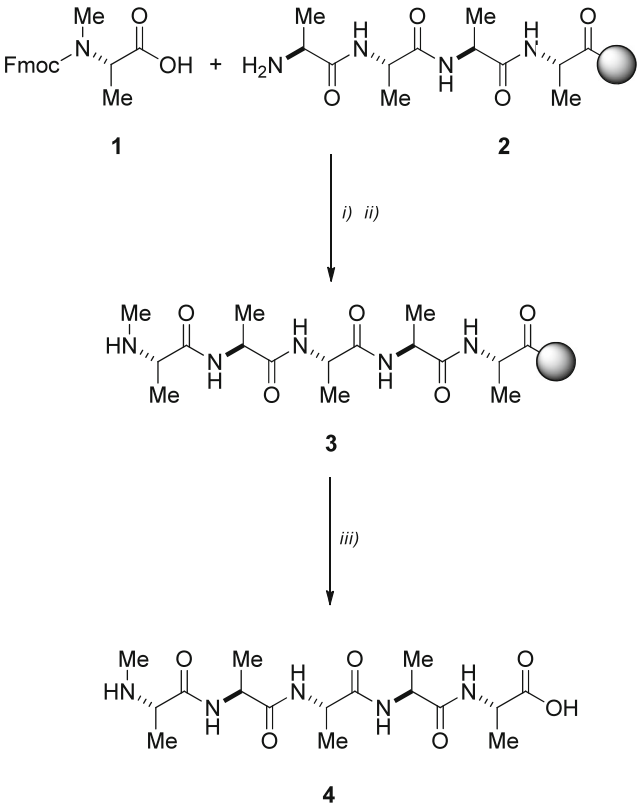


Fig. 1 Schematic illustration of the used CF apparatus

transfer, the efficient mixing of substrates, shorter reaction times and facile scale up [29–46].

In this work a fast and highly economic continuous-flow solid-phase peptide synthesis (CF-SPPS) technique is presented for the preparation of mono and multiple *N*-methylated oligopeptides. The technology ensures high yields for the desired peptides with the aid of readily available Fmoc-protected *N*-methylated amino acids applied in 1.5 M excess. Importantly, the method requires a considerably shorter time, while lower amount of waste is produced. Mono and multiple *N*-methylated oligoalanines and oligovalines were synthesized as model peptides including difficult sequences.

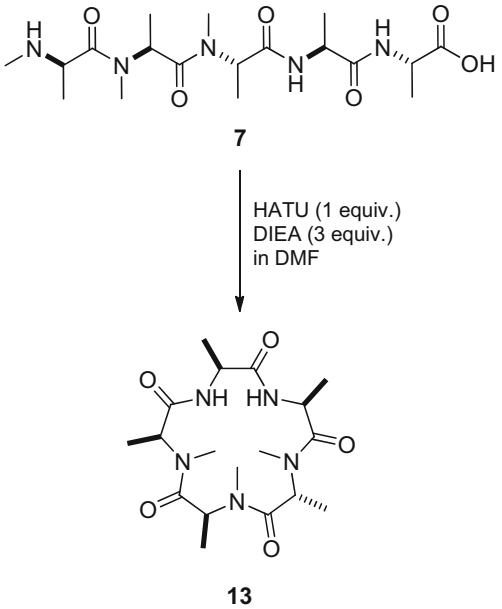


Scheme 1 The test reaction used for the fine-tuning of CF-SPPS coupling conditions. i) 1.5 equiv. of HATU, 3 equiv. of DIEA in DMF; ii) 2% DBU and 2% piperidine in DMF; iii) 90% TFA + 10% H2O

Results and discussion

Fmoc-protected *N*-methylated peptides are available on the market, thus their direct and cost-efficient incorporation into peptides is of considerable current interest. For this purpose, the highly efficient CF-SPPS method developed by our laboratory [35] is utilized and modified to ensure complete conversions for *N*-methylated sequences. The CF reactor used in this study (Fig. 1) consists of an HPLC pump, an HPLC autosampler, an HPLC column thermostat with a cylindrical PEEK column and a back-pressure regulator. For optimization of the CF-SPPS coupling conditions, an *N*-methylated amino acid, Fmoc-protected *N*-methyl alanine (*N*-Me-Ala) **1** was coupled to the Tentagel resin-bound alanine tetrapeptide **2**, with fine-tuning of the most important continuous-flow reaction parameters.

For the initial screening, the PEEK column was filled with 200 mg of **2**. It was placed into the column thermostat that can be heated up to a maximum of 100 °C. A back-pressure valve was fitted into the flow line to provide constant pressures up to



Scheme 2 The solution phase cyclization of *N*-methylated oligomer **7**

100 bar. The HPLC pump ensured a continuous stream of the reaction mixture at flow rates of 0.01–1.0 mL min^{-1}. 1.5 equivalents (equiv.) of the coupling reagent 1-[bis-(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*-]pyridinium-3-oxide (HATU) to form the active esters and 3 equiv. of *N,N*-diisopropylethylamine (DIEA) in 1 mL DMF was used to couple Fmoc-*N*-Me-Ala-OH to the resin-bound tetrapeptide **2** (Scheme 1). The deprotection step was performed by using 1 mL of deprotection solution (2% piperidine and 2% 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in *N,N*-dimethylformamide (DMF)). Between the two chemical steps, the system was washed for 10 min with DMF (Scheme 2).

A complete reaction parameter screening process was carried out, in order to attain the best conversion. First the temperature dependence of the coupling reaction was investigated. Increasing the temperature to 60 °C resulted in >90% conversion (Supporting Information, Table 1), but further increases led to lower conversions. This can be accounted for the degradation of the active ester [47]. Thus, 60 °C was taken as an optimal value. On examination of the pressure dependence at 60 °C, acceptable conversion was achieved at 80 bar (Supporting Information, Table 2). A flow rate effect scan was performed at 60 °C and 80 bar. The best result was obtained at 0.15 mL min^{-1} (Supporting Information, Table 3). To fine-tune the amount of the amino acid, the peptide synthesis was performed at 60 °C, 80 bar and 0.15 mL min^{-1}. 1.5 equiv. was satisfactory to maintain 97% conversion, but reducing this ratio resulted in poorer results (Supporting Information, Table 4). Finally, we turned our attention towards optimizing the concentration of the coupling medium. The results indicated that the lower the concentration of the coupling mixture, the higher the conversion. The test reaction was complete only at a concentration of 0.011 mM (Supporting Information, Table 5). On the basis of earlier results [35], the volume of the deprotection solution was 2 mL applied with a flow rate of 0.15 mL min^{-1}. The results of the reaction parameter optimization are shown in Fig. 2.

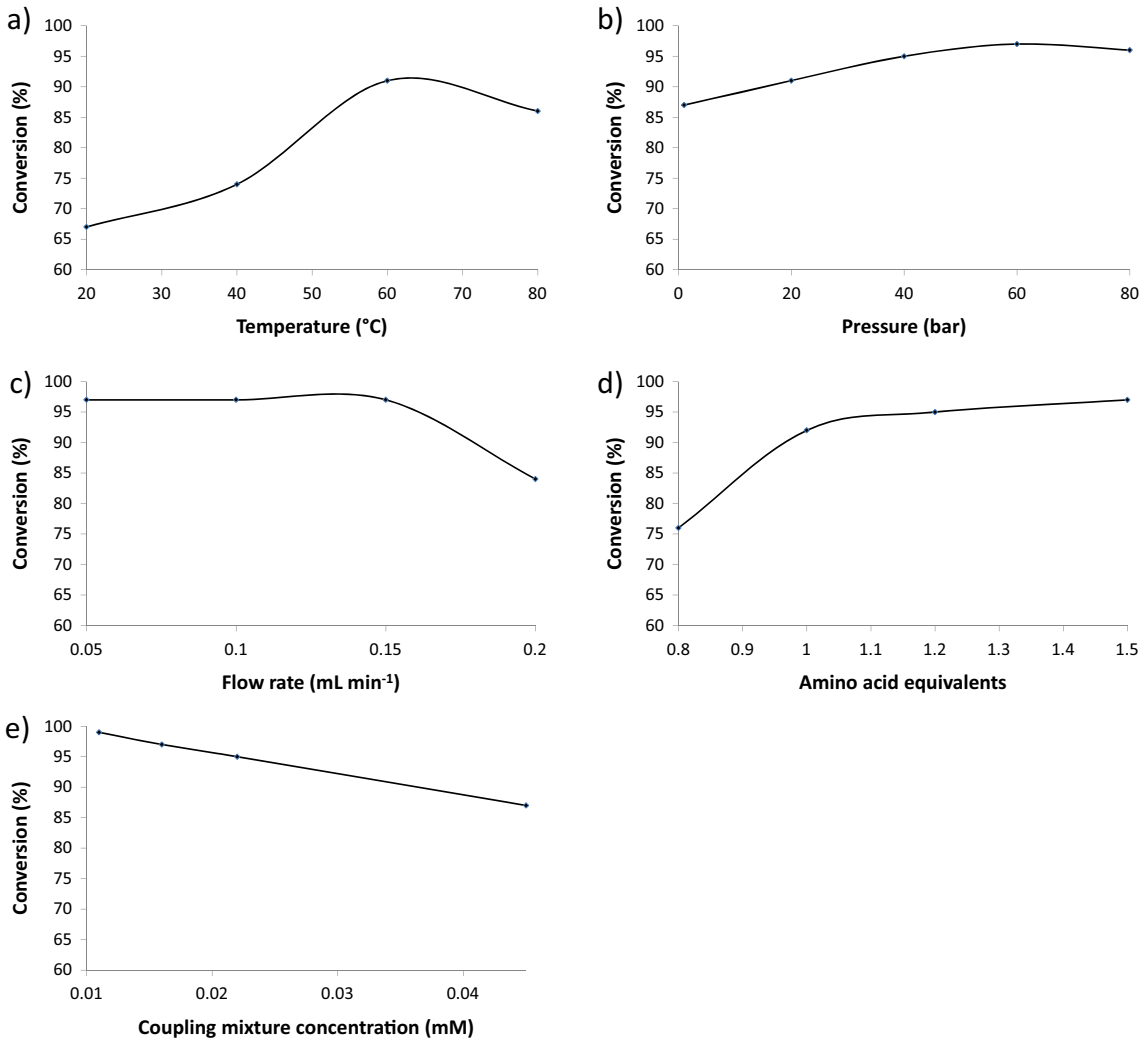


Fig. 2 The effect of temperature (**a**), pressure (**b**), flow rate (**c**), amino acid equivalents (**d**) and coupling mixture concentration on the conversion for the test reaction shown on Scheme 1

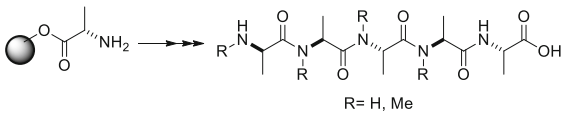
The optimized CF-SPPS technique might allow accomplishing the fast and powerful synthesis of numerous *N*-methylated peptides on solid support. As an evidence of the effectiveness of the procedure, mono and multiple *N*-methylated pentaalanine peptides were prepared. Oligoalanine peptides containing one or more methyl groups can be employed as template structures for designing biologically active compounds.

The *N*-methylated alanine pentapeptides were designed to have 1D- and 4 L–amino acids to afford a subsequent future efficient cyclization [26, 48]. Four different pentaalanine structures were created containing 1, 2, 3 or 4 *N*-methylated peptide bonds. The synthesized structures are shown in Table 1.

The Fmoc-Ala loaded resin was filled into the PEEK column. The coupling and deprotection steps were performed under the conditions optimized previously. After the synthesis the resin was transferred to a flask and the cleavage was carried out by the utilization of 90% trifluoroacetic acid (TFA) and 10% water. The crude peptides were analyzed by HPLC-MS and the yields are shown in Table 1. Importantly, the yields of the peptides are >90% and no truncated sequences were found in the product. The yields were calculated from resin loading. Outstandingly, no extra amino acid incorporation was detected resulting from the spontaneous deprotection of the activated amino acid. This fact can be explained by the short residence time of the coupling mixture spent on the resin bed compared to ordinary SPPS technologies and by the fast and efficient coupling of the amino acid to the peptide chain. The residence time was measured to be 5.8 min.

Since coupling efficiencies and raw purities were acceptable for alanine peptides, the technology was tested on difficult sequences. Peptides containing high number of valines residues are known as difficult sequences [49]. Thus pentavalines containing 1, 2, 3, and 4 *N*-methylated amino acid building blocks were also synthesized. The structure of pentavalines prepared is shown in Table 2. The synthesis of oligovalines **9–12** was carried out under the conditions optimized previously. Acceptable yield was achieved only for oligomer **9**, which contains only 1 *N*-methylated amino acid.

Table 1 The synthesized *N*-methylated oligoalanines and obtained yields



R= H, Me

coupling conditions: 1.5 equiv. Fmoc-AA-OH, 1.5 equiv HATU, flow rate: 0.15 mL min^{-1}, pressure 80 bar, temperature: 60 °C sample concentration: 0.011 mM

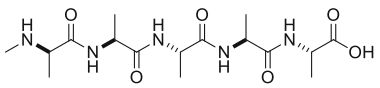
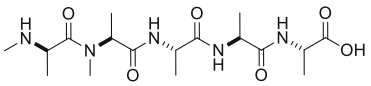
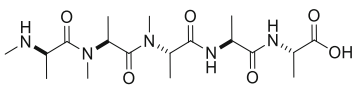
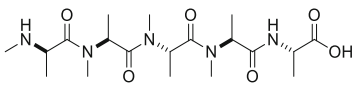
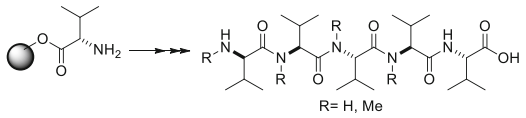
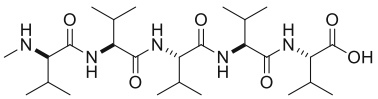
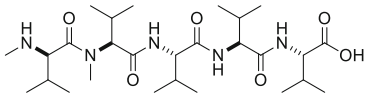
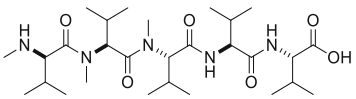
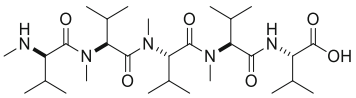
entry	structure	yield (%)
1	 **5**	>95
2	 **6**	94
3	 **7**	94
4	 **8**	92

Table 2 The investigated oligovalines and yields of the syntheses



coupling conditions: 1.5 or 2 equiv. Fmoc-AA-OH, 1.5 equiv HATU or Oxyma,
flow rate: 0.15 mL min^{-1}, pressure 80 bar, temperature: 60 °C sample concentration: 0.011 mM

entry	structure	crude purity (%)
1	 **9**	93
2	 **10**	>95[a]
3	 **11**	94[b]
4	 **12**	91[b]

[a] Oxyma was used as coupling agent

[b] Oxyma was used as coupling agent by the utilization of 2 equiv. of amino acids

For oligomer **10** the yield was considerably lower. Moreover, oligopeptides **11** and **12** were not isolated at all. Oxyma pure is known as a superior peptide coupling reagent compared to benzotriazole-based compounds [50]. Therefore, the synthesis of oligomers **10–12** was carried out by the utilization of 1.5 equiv. of oxyma pure and 1.5 equiv. of DIC as coupling agents in the presence of 1.5 equiv. of amino acids. This coupling strategy considerably increased the yield of the resulting oligomer **10** (>95%). However, for **11** and **12** it remained ~86% and ~78%, respectively, which is a reasonable value. To our delight, however, the yield could be further enhanced only by the utilization of 2 equiv. of the amino acids, providing the desired high values of 94% and 91% for oligomer **11** and **12**, respectively. Importantly no diketopiperazine formation was observed for peptides **8** and **12**, which contain *N*-methylated amino acids in the second position. The yields were calculated from resin loading.

N-methylated peptides mainly occur as cyclic peptides [26, 27, 48]. Therefore, oligomer **7** was selected for a cyclization experiment.

The synthesis of **7** was successfully scaled up to 0.15 mmol value and the peptide was isolated with 93% yield. The results of the synthesis are superior than those for the earlier ones (Table 3).

Importantly, our methodology required lower amounts of amino acid for coupling (1.5 equiv.) making the synthesis more cost-effective. Noteworthy, the coupling and deprotection times were 28 min under flow conditions, compared with 274 min in the former strategy. With this CF technique an amount of only 4.2 mL of solvent was used, as compared with the high solvent consumption (66 mL) of the original approach. Importantly, 3 additional chemical steps were avoided. Importantly, the technology is not only faster, but considerably more sustainable.

Table 3 Comparison of the results of the syntheses of **7** with literature data [48]

Property	CF-SPPS	Literature [48]
Yield	93%	n.d.
Amino acid equivalents	1.5	3
Coupling and deprotection times	28 min	274 min
Solvent used	4.2	66

Easy and effective solution-phase cyclization of peptides can be carried out by means of a dual syringe pump utilizing the pseudo-high dilution peptide cyclization technology [51–54]. In a typical experiment, a solution of 1 equiv. of linear peptide in 10 mL DMF was transferred into a syringe. To the equal volume of DMF 1 equiv. of HATU was added and poured into another syringe. Both reaction mixtures were infused into a vigorously stirred solution of 3 equiv. of DIEA in 40 mL DMF at a flow rate of 0.015 mL min^{-1}. After completion, the mixture was stirred for 30 min. The reaction mixture was diluted with 50 mL water and lyophilized. The conversion and the purity of the crude cyclic peptide was determined by RP-HPLC-MS analysis. The conversion of the cyclization experiment was quantitative and the RP-HPLC purification provided 68% yield from resin loading for peptide **13**. It is a considerably higher value than the published result (26%) [48].

Conclusion

In summary, a rapid, simplified and highly efficient CF-SPPS technology is reported for the direct synthesis of mono and multiple *N*-methylated cyclic alanine and valine peptides. Through an optimization study, we find that in general only 1.5 equivalents of the amino acids are sufficient for the couplings to maintain excellent conversions. This is a cheap and time-efficient approach requiring low amounts of solvents and reagents. Furthermore, it is also applicable to the coupling of challenging amino acids. Importantly, the peptides were synthesized directly, avoiding the on-resin methylation of the amino acids, which allows shortening the synthesis by three chemical steps. Mono and multiple *N*-methylated pentaalanines and pentavalines were prepared as model and difficult sequences respectively. In the case of alanines, the results were satisfactory under the optimized conditions. To obtain excellent crude purities of pentavalines, however, required the use of oxyma pure as coupling agent and 2 equivalents of amino acids. A scaled-up synthesis was also performed and a subsequent cyclization of the peptide utilizing the pseudo-high dilution solution-phase technology was carried out too.

Experimental (materials and methods)

Peptide synthesis: The linear peptide chains were extended on a Tentagel R PHB resin (0.20 mmol g^{-1}). For CF experiments, a modular CF apparatus was assembled, consisting of a cylindrical PEEK column (with internal dimensions of 100 × 4 mm) filled with the amino acid loaded resin (200 mg), an HPLC pump (JASCO PU-880), an HPLC autosampler (JASCO AS-2055 Plus), an HPLC column thermostat (Dionex STH 585), and a backpressure regulator (Upchurch Scientific/IDEX Health & Science P-465 PEEK). A coupling mixture consisting of 1.5 equivalents of Fmoc-protected amino acid and 1.5 equivalents of HATU as coupling reagent dissolved in 1 mL DMF and 3 equivalents of DIEA was mixed by the autosampler. The coupling mixture has been prepared just before the coupling reaction. The coupling reactions were carried out at the optimized reaction conditions, 80 bar pressure, 60 °C temperature, 0.15 mL min^{-1} flow rate. For Fmoc deprotection 2 mL of 2% DBU 2% piperidine DMF solution has been used. Between two chemical steps for washing DMF has been used for 6 min long with 0.15 mL min^{-1} flow rate.

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